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THE EFFECTS OF SURFACTANTS ON CELL AGGREGATION

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SUMMARY

Trypsin-dissociated HeLa and human amnion cells were employed to investigate the effects of an anionic, a cationic, and a non-ionic surfactant on cell reaggregation. All the surfactants inhibited the reaggregation process, the effect increasing with surfactant concentration, and becoming significant at concentrations of about 10^{-4} M for the anionic surfactant and about 10^{-3} M for the others. The effects of temperature and ionic strength on HeLa cell aggregation, and of surfactants on the electrophoretic mobility of HeLa cells and the release of sialic acid from their surfaces by neuraminidase, were also examined. The results indicate that although electrostatic effects occur and are likely to be of importance in cell reaggregation, the effect of surfactants cannot be accounted for on this basis alone, and it is suggested that interactions involving the cell-surface glycoproteins, possibly indirectly via effects on the fluidity of the cell membrane, are also significant in this respect.

INTRODUCTION

Surfactants are molecules which, because of their amphiphilic structure, are absorbed at surfaces, modifying their properties. That they interact with biological surfaces and macromolecules has long been known, and in particular their effectiveness in disaggregating embryonic and other biological tissue has been reported (Yamada, 1962; Devillers, 1968; Pearce & Grimmer, 1972). Their interaction with the cytoplasmic membrane has mainly been investigated by means of many studies of the haemolysis of erythrocytes and the solubilization of the erythrocyte membrane (e.g. Pethica & Schulman, 1953; Bonsall & Hunt, 1971). Some work has also been done on the effect of surfactants on the release of histamine from mast cells (Prottey & Ferguson, 1976) and on the lysis of tumour cells (Hodes, Palmer & Livengood, 1961). This work, which has been interpreted in terms of the action of the surfactants on the lipid regions of the cytoplasmic membrane, has been essentially concerned with the cytotoxic effect of relatively high concentrations of surfactants, though recently (Ferguson & Prottey, 1976) some measurements of the effect of sublethal concentrations of surfactants on DNA synthesis in fibroblasts have been reported. There have, however, been no systematic studies of the influence of surfactants on intercellular phenomena such as cell adhesion.

Apart from the practical importance of such studies, comparisons of the effects of surfactants on cell adhesion with their effects on other surface properties, might

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provide an indication of which surface properties are important for cell adhesion. We have therefore measured the influence of an anionic, a cationic and a non-ionic surfactant on the aggregation of cells, on electrophoretic mobility, and on the release of sialic acid by neuraminidase from the cell surface glycoproteins, and also for comparison, the effects of temperature and ionic strength on the aggregation process.

MATERIALS AND METHODS

Cell culture and dissociation

The cell lines employed were HeLa S3 and Human Amnion FL supplied by Flow Laboratories, Irvine, Scotland. They were grown as confluent monolayers in culture bottles at 37 °C in Eagle's MEM containing 11 % bovine serum for the HeLa cells and 13 % calf serum for the amnion cells.

The confluent monolayers were detached from the glass surface of the culture bottles as follows. Ten millilitres of a 0·25 % solution of trypsin (1:250) in 154 mM saline were added to the bottle to cover the monolayer of cells. After 2 min the trypsin was removed and the cells left a further 15 min after which they are easily detached from the glass surface. They are then suspended in Earle's BSS (Ca and Mg free) for 30 min during which time complete dissociation into single cells occurs. These are removed by centrifugation and resuspended at a concentration of 3×10^5 cells/ml in the medium in which aggregation is to take place.

Aseptic conditions were maintained throughout dissociation and subsequent aggregation procedures.

Reaggregation

The surfactants used in these studies were sodium dodecyl sulphate (SDS) (B.D.H. Ltd, specially pure), cetyl trimethyl ammonium bromide (CTAB) (Palmer Research Laboratories Ltd) and Tergitol 15-S-7 (Union Carbide Corporation). The last is a commercial mixture of non-ionic ethylene oxide surfactants. The average molecular weight is taken as being 500.

Aggregation was measured in Eagle's MEM to which various amounts of surfactant had been added, except for the purpose of studying the effect of ionic strength on aggregation. For this HeLa cells were suspended in saline (0·15 M NaCl) mixed with 0·3 M sucrose to give isosmotic solutions with ionic strengths of 150, 100, 75 and 50 mM.

Aggregation studies were performed using the gyratory shaker technique (Moscona, 1961*a*; Kemp, 1970). Sealed Erlenmeyer flasks containing 3-ml aliquots were shaken in a water bath at 70 rev/min. To maintain the pH of the medium at 7·6 throughout the course of the experiment, the flasks were gassed with a mixture of 5 % CO_2 in air (Paul, 1965). The cell suspensions were shaken for periods of up to 4 h. Experiments were carried out at 37 °C except for a few at temperatures down to 25 °C.

The degree of aggregation which had taken place in the shaker after a given time was estimated by examining samples of the suspension in a modified Fuchs–Rosenthal haemocytometer, and measuring the number of single cells remaining. The trypan blue staining technique was used to indicate cell viability during the shaker experiments. Preliminary results indicated that in order to obtain a meaningful measure of aggregation from the shaker experiments, it was necessary to investigate the effect of shear rate on the process. For this purpose reaggregation of HeLa cells was measured in a Couette type viscometer (Curtis, 1969; Hornby, 1971). A Haake viscometer (Rotovisko) was modified by having a spiral groove cut in its inner cylinder to prevent sedimentation. In this instrument shear rates of between 0·05 s^{-1} and 100 s^{-1} could be obtained. The aggregation of HeLa cells at 37 °C in Eagle's MEM was measured.

Electrophoresis

HeLa cells obtained from monolayers as described above, were suspended in calcium- and magnesium-free BSS for 1 h at 37 °C in the presence or absence of surfactant. The cells were removed by centrifugation and resuspended in 0·145 M NaCl solution containing 3×10^{-4} M

sodium bicarbonate. The electrophoretic mobilities of the cells were measured at 25 °C in a cylindrical cell apparatus (Bangham, Flemans, Heard & Seaman, 1958; Seaman, 1963) manufactured by Rank Bros. Ltd.

Sialic acid release

HeLa cell suspensions in calcium- and magnesium-free BSS were incubated for 1 h in the presence of surfactants at 37 °C. The cells were removed by centrifugation, resuspended in 1 ml of calcium- and magnesium-free BSS and incubated with 0·05 ml of neuraminidase solution (500 units per ml neuraminidase ex *Vibrio cholerae* from BDH Chemicals Ltd.), at 37 °C for 1 h. The amount of sialic acid liberated was estimated by the thiobarbituric acid method (Warren, 1959).

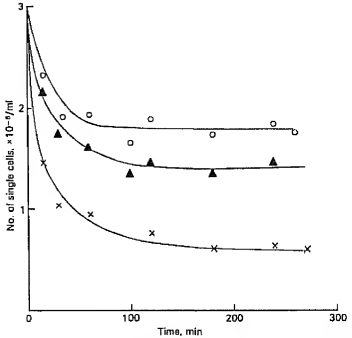

Fig. 1. The effect of CTAB on the aggregation of HeLa cells in a rotatory shaker. The number of single cells remaining is shown as a function of time in the presence of (×) no CTAB, (▲) 2×10^{-5} M CTAB and (○) 4×10^{-5} M CTAB.

RESULTS

Reaggregation of HeLa cells

The time course of the reaggregation of HeLa cells is shown in Fig. 1(×) in which the number of single cells remaining is shown as a function of time. The single cell count fell to about 67% of its initial value in 60 min and after this much more slowly, reaching a steady value after about 2 h. No sign of a trypsination lag (Steinberg, Armstrong & Granger, 1973) was observed. The viability of the cells was above 95% at all times. The aggregates formed were relatively large and smooth and round in appearance (Fig. 6, p. 370). It has been shown by Hornby (1973) that on the basis of

the Smoluchowski relationship for flocculation under random motion (Swift & Friedlander, 1964), the fall in numbers of single particles in a given time should be given by

$$\frac{1}{\sqrt{(N_o N_t)}} = \frac{1}{N_o} + aYt, \qquad (1)$$

where N_o is the number of single particles at $t = 0$, N_t is the number of single particles at time t, a is the collision efficiency – a measure of adhesiveness (Curtis, 1969), and Y is a rate constant characterizing random motion in the shaker system. Hornby

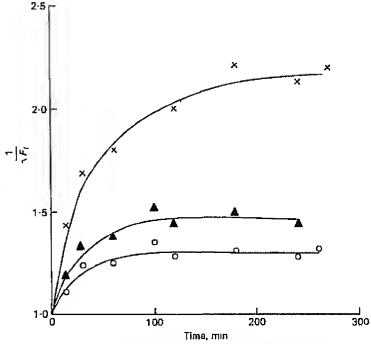

Fig. 2. The aggregation of HeLa cells in a rotatory shaker. The variation of $1/\sqrt{F_t}$ with time, in the presence of (×) no CTAB, (▲) 2×10^{-6} M CTAB, and (○) 4×10^{-6}M CTAB, where F_t is the fraction of single cells remaining at time t.

showed that the aggregation of chick embryo cells obeyed equation 1 for periods of at least 2·5 h after the beginning of aggregation. However when our results for HeLa cells are plotted in the form $1/\sqrt{F}$ against t where $F_t = N_t/N_o$ it will be seen from Fig. 2 (×) that a linear relationship cannot be said to be maintained for more than about 1 h, after which the variation of $1/\sqrt{F_t}$ with t decreases and soon becomes zero. This limit to aggregation could be explained in terms of a low limit to the number of adhesive sites per cell for these particular cells, but as the aggregates formed in general contain many cells, it is more likely that the limit is due to shearing forces.

It was suspected that the situation envisaged by Curtis (1969) was occurring, in which the shearing forces in the shaker were not only bringing cells into contact but were also removing cells from aggregates, a possibility for the relatively weakly ad-

hering HeLa cells. To test this hypothesis the effect of shear rate on the reaggregation process was investigated, by means of measurements of aggregation in a Couette viscometer in which linear shear at known and controlled rates could be applied. Dissociated HeLa cells were reaggregated in Eagle's MEM. The variation of the number of single cells remaining with time was measured at various shear rates. For shear rates of less than 0·5 s^{-1} it was necessary to add 2·15 % of sucrose to the medium to increase viscosity and eliminate sedimentation. The results are shown in Fig. 3 in

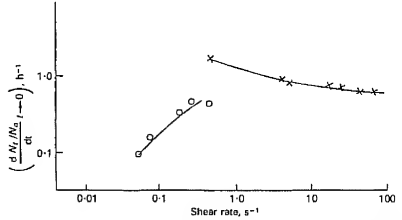

Fig. 3. The aggregation of HeLa cells in a Couette viscometer. The initial rate of decrease in the number of single cells $[d(N_t/N_0)/dt]_{t\to 0}$, as a function of shear rate (×). The results in curve (○) were obtained in the presence of 2·15 % sucrose.

which the initial rate of decrease of single cells $[d(N_t/N_0)/dt]_{t\to 0}$; is shown as a function of shear rate. This initial rate of decrease of single cells increases linearly with shear rate up to a shear rate of about 0·2 s^{-1} as would be predicted by Smoluchowski's theory for the coagulation of a suspension under laminar shear conditions (Smoluchowski, 1916). At higher shear rates however the initial rate of decrease becomes independent of shear rate or even decreases slightly as shear rate is increased. The Smoluchowski theory assumes that the shearing forces serve only to bring the particles together and not to break up aggregates. If this assumption is not justified, and if the breaking up process becomes important during the time of the first few measurements of cell numbers, then a true initial rate will not be observed but some lower value which will depend on the balance of aggregation and breaking up rates. This appears to be happening for shear rates greater than 0·2 s^{-1}.

Comparisons of aggregation rates indicates that the (non-laminar) shear conditions occurring in the shaker are the equivalent of greater shear rates than 0·2 s^{-1}. However apparent initial aggregation rates can be determined and the initial slope of the $1/_4F_t$ vs t curve should give a good relative measure of adhesiveness in the initial stages of aggregation, though the calculation of actual adhesive forces from it would not be justified.

In all cases the system reached an equilibrium level at which the number of single

cells did not vary with time at a measurable rate. At this stage the rate of removal of single cells from the system by aggregation will be equal to the rate of formation of single cells by the break up of aggregates. Under these circumstances $(N_o - N_a)/N_o$ should be a satisfactory relative measure of cell adhesion, where N_o is the initial number of single cells and N_a is the number of single cells at equilibrium.

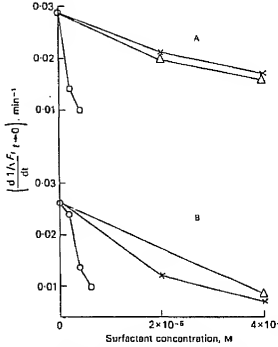

Fig. 4. The initial rate of aggregation $[d(1/\sqrt{F_t})/dt]_{t\to 0}$, min^{-1} of A, human amnion cells and B, HeLa cells as a function of surfactant concentration (○) for SDS, (×) for CTAB, and (△) for Tergitol.

The effects of surfactants on the aggregation of human amnion and HeLa cells

Figs. 4 and 5 show the effects of various concentrations of surfactants on cell re-aggregation. For amnion cells, the effect of surfactant concentration on the initial slope of the $1/\sqrt{F_t}$ vs t curve is shown in Fig. 4A and on the situation at equilibrium, $(N_o - N_a)/N_o$ in Fig. 5A. Figs. 4B and 5B show the same relationships for HeLa cells. Each result was obtained as the mean of 5 aggregation experiments. The cell viability did not fall below 90% except for systems containing the highest concentration of SDS (6×10^{-5} M) for which it was 85% at the end of the experiment. For both cell lines the aggregates formed in the presence of surfactants were always smaller, fewer in number and in general less compact than in controls. Fig. 7 shows the aggregates formed after 3 h in 2×10^{-5} M CTAB.

The effect of temperature and ionic strength on the aggregation of HeLa cells

Decreasing the temperature results in a substantial reduction in reaggregation rate as is shown in Table 1.

Table 2 shows that a reduction in ionic strength results in reduction in reaggregation.

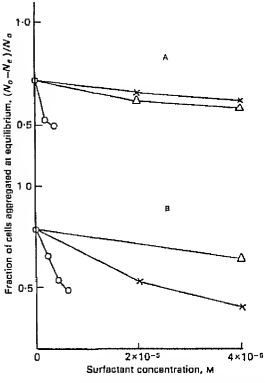

Fig. 5. The equilibrium aggregation of (A), human amnion cells and B, HeLa cells as a function of surfactant concentration (○) for SDS, (×) for CTAB, and (△) for Tergitol.

Table 1. *The effect of temperature on the reaggregation of HeLa cells in Eagle's MEM*

Temperature, °C	37	34	30	25
Fraction of cells aggregated in 60 min, $1-N_{60}/N_0$ where N = single cell count	0·64	0·60	0·58	0·32

The effect of surfactants on the electrophoretic mobility of HeLa cells

The electrophoretic mobility of HeLa cells in 0·145 M NaCl, 3×10^{-4} M $NaHCO_3$ solution was $1\cdot03\pm0\cdot04$ $\mu m\ s^{-1}\ V^{-1}$. After treatment with the surfactants, the mobility became very variable. For example cells treated with 2×10^{6} M SDS, the anionic surfactant, had mobilities ranging from 1·26 to 1·88 $\mu m\ s^{-1}\ V^{-1}$ but always higher than

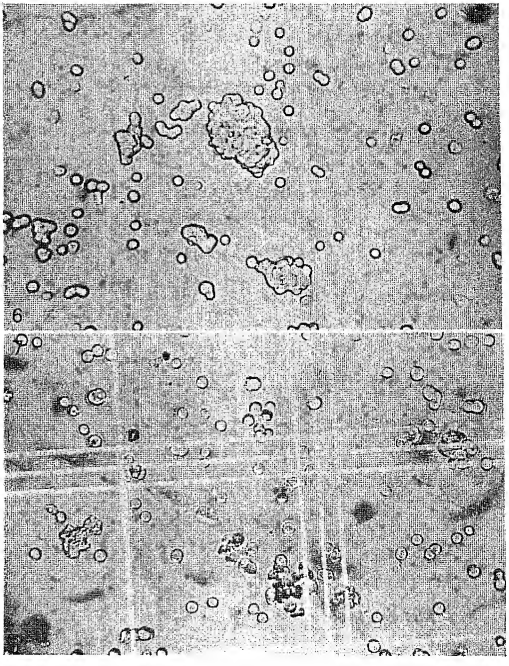

Fig. 6. Aggregates formed by HeLa cells after 3 h in the absence of surfactant.
Fig. 7. Aggregates formed by HeLa cells after 3 h in 2×10^{-6} M CTAB.

for untreated cells. Cells treated with 1×10^{-5} M CTAB, the cationic surfactant, had mobilities ranging from 0·98 to 1·18 $\mu m\ s^{-1}\ V^{-1}$ and could not be said to have a significantly different mobility from the untreated cells.

The effect of surfactants on the release of sialic acid from cell surfaces by neuraminidase

The amount of sialic acid which can be released from HeLa cell surfaces by treatment with neuraminidase depends on the previous history of the cell and in particular the dissociation procedure used. Immediately after vigorous trypsinization, virtually no sialic acid is released. For the mild trypsinization conditions described followed by a recovery period, 1·16 nmol are obtained from 10^6 HeLa cells. If the cells are in a medium containing surfactant during the recovery period the amount of sialic acid released by subsequent neuraminidase treatment is enhanced as shown in Table 3.

Table 2. *The effect of ionic strength on the reaggregation of HeLa cells*

Ionic strength, mM	150	100	75	50
Fraction of cells aggregated in 60 min, $1 - N_{60}/N_0$ where N = single cell count	0·36	0·2	0·12	0·08

Table 3. *The effect of surfactants on the amount of sialic acid released from HeLa cells by neuraminidase treatment*

Surfactant	% enhancement of sialic acid release
Anionic (SDS), M	
2×10^{-6}	35
4×10^{-6}	121
5×10^{-6}	179
Cationic (CTAB), M	
1×10^{-5}	34
3×10^{-5}	42

DISCUSSION

Before the reaggregation of cells can be studied the cells must be dispersed and there is much evidence that the particular agent used – trypsin or EDTA – and the conditions employed, can have a substantial effect on the course of the subsequent reaggregation process (Steinberg *et al.* 1976; Lloyd, Rees, Smith & Judge, 1976; McGuire, 1976). Dissociation can result in the modification or destruction of surface structures which must be restored before effective adhesion can take place. This is particularly evident when vigorous trypsinization is used to obtain cells from coherent tissue. Under these circumstances a trypsinization lag in reaggregation is observed (Steinberg *et al.* 1973). The dissociation procedure used in the present work gives no such lag period, and although there is no doubt that some disturbance of the cell

surface and removal of material must occur (Lloyd *et al.* 1976), it is evident that surface conditions are adequately restored before the aggregation measurement is commenced.

Even in the absence of surfactant, the HeLa and human amnion cells adhere weakly compared, for example with the chick embryo cells studied by Hornby (1973). The rapid attainment of an equilibrium situation in which a large fraction of single cells are present, indicates that under the shear conditions in the shaker, adhesion is reversible over the 4-h period studied. The course and extent of reaggregation are quite similar for the 2 cell lines as is indicated by the results for zero concentration of surfactant shown in Figs. 4 and 5.

The effect of ionic strength on reaggregation is shown in Table 2. The results are obtained from measurements made over periods of up to 75 min as cell viability fell below acceptable levels over longer periods in the media used, which were, necessarily, quite different from that used in the rest of the work. Thus the results refer essentially to short-term effects. It is clear that an increase in ionic strength is associated with an increase in aggregation. From this it can be concluded that charge effects are important in the initial stages of aggregation of these cells. The increase of adhesion with ionic strength would be predicted on the basis of the simple hydrophobic colloid flocculation theory applied to cell aggregation (Curtis, 1962) as a reduction in the extent of the diffuse double layer leading to lower repulsive forces, but other explanations of the effect are possible. For example local short-range electrostatic interactions between charged groups on the surfaces of adhering cells would be affected. Also interactions between charged groups on the same cell surface would be reduced, with resultant changes in the alignment conformation and mobility of cell surface macromolecules.

The observed reduction in cell aggregation as temperature is lowered confirms many previous observations (Zeidman, 1949; Moscona, 1961*b*; Orr & Roseman, 1969; Attramadal & Jonsen, 1974). The usual explanation for this phenomenon is in terms of a reduction in metabolic activity in the cell resulting in the slowing down of the synthesis of materials necessary for adhesion, but here too alternative explanations are possible (Ueda, Ito, Okada & Ohnishi, 1976). A reduction in temperature will reduce molecular mobility in the lipid bilayer parts of the membrane and may produce changes in the mesomorphic forms present in these regions. The distribution of receptor sites on the cell surface is altered by low-temperature treatment (Smith & Revel, 1972). Recently it has been shown that changes in the cell membrane lipid of the kind that would result in changes in membrane fluidity produce changes in cell adhesion (Curtis, Chandler & Picton, 1975).

Figs. 1, 2, 4 and 5 show the effect of surfactants on cell reaggregation. The particular concentrations of each surfactant used were those which produced measurable effects on cell adhesion without reducing cell viability. Clearly this range occurs at far lower concentrations for the anionic surfactant, SDS (10^{-6} M), than for the others (10^{-5} M). However, the presence of surfactant always inhibits the reaggregation process as measured by the reduction in the number of single cells and this is confirmed by the visual effect of surfactants on the size and number of aggregates. Com-

parison of Fig. 4A with B and of Fig. 5A with B shows that the effect of surfactants on the 2 cell lines is very similar, and indeed all previous work (Yamada, 1962; Devillers, 1968; Pearce & Grimmer, 1972) has indicated that in general all surfactants reduce adhesion for all cell lines and tissues examined and that some mechanism which is not specific to any one surfactant or cell type is involved. Comparison of Fig. 4, which is concerned with initial rates of reaggregation with Fig. 5 which is derived from the equilibrium state reached after 3–4 h in the shaker shows that surfactants have much the same effect on initial as on final rates. This indicates that although surfactants are known to affect cell metabolism (Ferguson & Prottey, 1976), this is not the only means by which they reduce cell adhesion.

The electrophoresis measurements show that incubation with surfactants greatly increases the variation of electrophoretic mobility in a population of cells. A similar phenomenon has been noted for cells treated with versene (Hayry, Pentinnen & Saxen, 1965). It seems likely that changes in the arrangement of charged groups relative to the surface of shear may be involved but clearly further investigation is required to determine the cause of this behaviour. Nevertheless it is suggested that the effect of surfactants might be thought of as converting a population of cells, relatively homogeneous as far as surface properties are concerned into a comparatively heterogeneous population. The effect of this might be to inhibit aggregation by a reduction in the specificity of the surface.

Cells treated with the anionic surfactant SDS (10^{-9} M) had a greatly increased negative potential whereas treatment with the cationic surfactant CTAB (10^{-5} M) sometimes increased, sometimes decreased the zeta potential. At these concentrations the surfactants had a comparable effect in inhibiting cell reaggregation. Thus, although the variation of aggregation rate with ionic strength indicates that electrostatic forces are involved in some way with cell adhesion, clearly the effects of surfactants is not simply a matter of adsorption of charged molecules on the cell surface leading to a change in overall surface potential, and thus to a change in electrostatic repulsion forces between cells. Such a mechanism is possible for the anionic but not for the cationic or non-ionic surfactants. Similarly a mechanism in which surfactant molecules reduced cell adhesion by providing alternative binding sites for divalent ions could only be possible for the anionic compound.

There is a great deal of evidence that cell surface glycoproteins are involved in cell adhesion (Kemp, Lloyd & Cook, 1973). It is also known that surfactants interact with glycoproteins to form complexes (Weber, Pringle & Osborn, 1972). Table 3 shows that both anionic and cationic surfactants enhance sialic acid release from the cell surface by neuraminidase, the effect of 10^{-9} M SDS being similar to that of 10^{-5} M CTAB. In a general sense this behaviour is parallel to the effect of the same surfactants on cell reaggregation, and suggests that surfactants interact with surface macromolecules in a way which makes them less able to mediate cell adhesion, but renders them more open to attack by neuraminidase. It cannot, however, be assumed that these effects are the result of a direct interaction between the surfactant and the macromolecule. All the measurements on the lytic effects of high concentrations of surfactants have been adequately interpreted in terms of interactions between the sur-

factants and the cytoplasmic membrane lipids (Bonsall & Hunt, 1971; Prottey & Ferguson, 1976).

There is direct evidence that the incorporation of surfactants into phospholipid systems related to membranes results in changes in molecular mobility (Ribeiro & Dennis, 1976), and that SDS influences the lipid/protein interactions in the rat liver membrane (Bont, Emmelot & Vazdias, 1969). Changes in molecular mobility in the cytoplasmic membrane have been related to changes in cell adhesion (Ueda *et al.* 1976; Curtis *et al.* 1975). Consequently it is possible that surfactants affect cell adhesion by changing membrane lipid fluidity, or mesomorphic form.

The effect of lowering the temperature, which reduces molecular motion in the membrane as measured by an ESR probe technique, and reduces aggregation (Ueda *et al.* 1976) and the effect of altering the fatty acid composition of the membrane in which increased unsaturation decreases aggregation (Curtis *et al.* 1975) appear at first sight to be contradictory, but it should be emphasized that concepts such as fluidity or viscosity are strictly only applicable to bulk phases and the relationships between fluidity, molecular translation and rotation, and interactions between molecules which apply to bulk phases do not necessarily apply exactly to biological membranes, so that the effect of altering temperature and molecular composition on these relationships may be different for the 2 systems. Also it is now generally accepted that cell adhesion is a complex phenomenon with a multiplicity of stages in some of which factors favoured by membrane fluidity – e.g. the formation of pseudopodia (Pethica, 1961) increase adhesion, while in others adhesion is improved by increased rigidity (Weiss, 1967; Rees, Lloyd & Thom, 1977). The little evidence available indicates that surfactants are likely to increase molecular motion in membranes (Ribeiro & Dennis, 1976; Van Zutphen, Merola, Brierley & Cornwell, 1972) and this is supported by their action in facilitating the penetration of membranes by drugs (Gillan & Florence, 1973).

Thus if their effect on cell adhesion is a result of alterations in membrane fluidity this is entirely compatible with the effect of changing fatty acid composition (Curtis *et al.* 1975). However, it should be emphasized that surfactants appear to be non-specific in their effects on biological systems, and their action on surface macromolecules and on cell adhesion is likely to be the result of a number of interactions.

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